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Defining the Phosphodiesterase Superfamily Members in Rat Brain Microvessels

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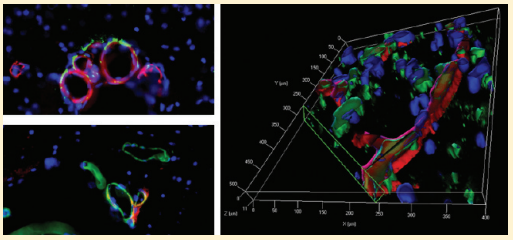
ABSTRACT: Eleven phosphodiesterase (PDE) families are known, each having several different isoforms and splice variants. Recent evidence indicates that expression of individual PDE family members is tissue-specific. Little is known concerning detailed PDE component expression in brain microvessels where the blood-brain-barrier and the local cerebral blood flow are thought to be regulated by PDEs. The present study attempted to identify PDE family members that are expressed in brain microvessels. Adult male F344 rats were sacrificed and blocks of the cerebral cortex and infratentorial areas were dissected. Microvessels were isolated using a filtration method, and total RNA was extracted. RNA quality and quantity were determined using an Agilent bioanalyzer. The isolated cortical and infratentorial microvessel total RNA amounts were 2720 ± 750 ng (n = 2) and 250 ± 40 ng (n = 2), respectively. Microarrays with 22 000 transcripts demonstrated that there were 16 PDE transcripts in the PDE superfamily, exhibiting quantifiable density in the microvessels. An additional immunofluorescent study verified that PDE4D (cAMP-specific) and PDE5A (cGMP-specific) were colocalized with RECA-1 (an endothelial marker) in the cerebral cortex using both F344 rats and Sprague−Dawley rats (n = 3−6/strain). In addition, PDE4D and PDE5A were found to be colocalized with alpha-smooth muscle actin which delineates cerebral arteries and arterioles as well as pericytes. In conclusion, a filtration method followed by microarray analyses allows PDE components to be identified in brain microvessels, and confirmed that PDE4D and PDE5A are the primary forms expressed in rat brain microvessels.



KEYWORDS: Brain microvessel, phosphodiesterase, microarrays, Immunofluorescent labeling, rat

Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that regulate the cellular levels of the second messengers, cAMP and cGMP, by controlling their rates of degradation. The functions of the PDEs include modulation of vascular tone and permeability to ensure an appropriate blood supply and molecular delivery to local tissues. Eleven PDE families have been identified, each having several different isoforms and splice variants.[1] On the other hand, expression of individual PDE family members may be tissue-specific. Following the well-accepted definition for nomenclature, a PDE family member written in lower case letters in *italics* refers to the nucleotide while those written in non-italicized capital letters refer to the protein.[1] Expression of the cGMP-stimulated PDE2A is differential in human venous and capillary endothelial cells.[2] PDE4a and PDE4b express at both transcriptional and translational levels; simultaneously, *pde4d* mRNA is detectable but PDE4D protein cannot be identified with the isoform-specific antibody in rat pulmonary microvascular endothelial cells.[3] Activities of the cAMP PDE and the cGMP PDE were identified in bovine brain microvessels three decades ago,[4] and more recently the expression of PDE1 and PDE5 was characterized in guinea pig basilar arteries.[5] Nevertheless, there is limited information concerning detailed expressions of the PDE superfamily components in brain microvessels.

The brain-blood-barrier (BBB) maintains the homeostasis of the brain. Studies using dyes and electron-dense tracers indicate that the primary BBB occurs at the level of the intracerebral microvasculature.[6] Vascular endothelial cells at the blood−brain interface are sealed together at their edges by tight junctions and make up the walls of microvessels. The tight junctions of these cells are composed of smaller transmembrane protein subunits, frequently biochemical dimers. There is increasing evidence that PDEs may control the BBB through modulating cAMP or cGMP levels. Elevation of intracellular cAMP levels increases the electrical resistance of endothelial monolayers by stabilizing intercellular junctional complexes[7] and thus reduces BBB permeability. Decreased cAMP and increased calcium levels are linked to increased permeability in the endothelial cells.[8−10] Conversely, agents that increase endothelial cAMP levels prevent barrier injury in the lung injury models.[11,12] Evidence from other organs, such as kidney, suggests PDE involvement in osmotic water permeability. PDE function underlies arginine vasopressin-regulated water reabsorption in the principal cells of the renal collecting duct by terminating protein kinase A (PKA) signaling through hydrolysis of localized cAMP.[13] A recent report indicates that PDE4D tethers EPAC1 in a vascular endothelial cadherin (VE-Cad)-based signaling complex and controls cAMP-mediated vascular permeability.[14]

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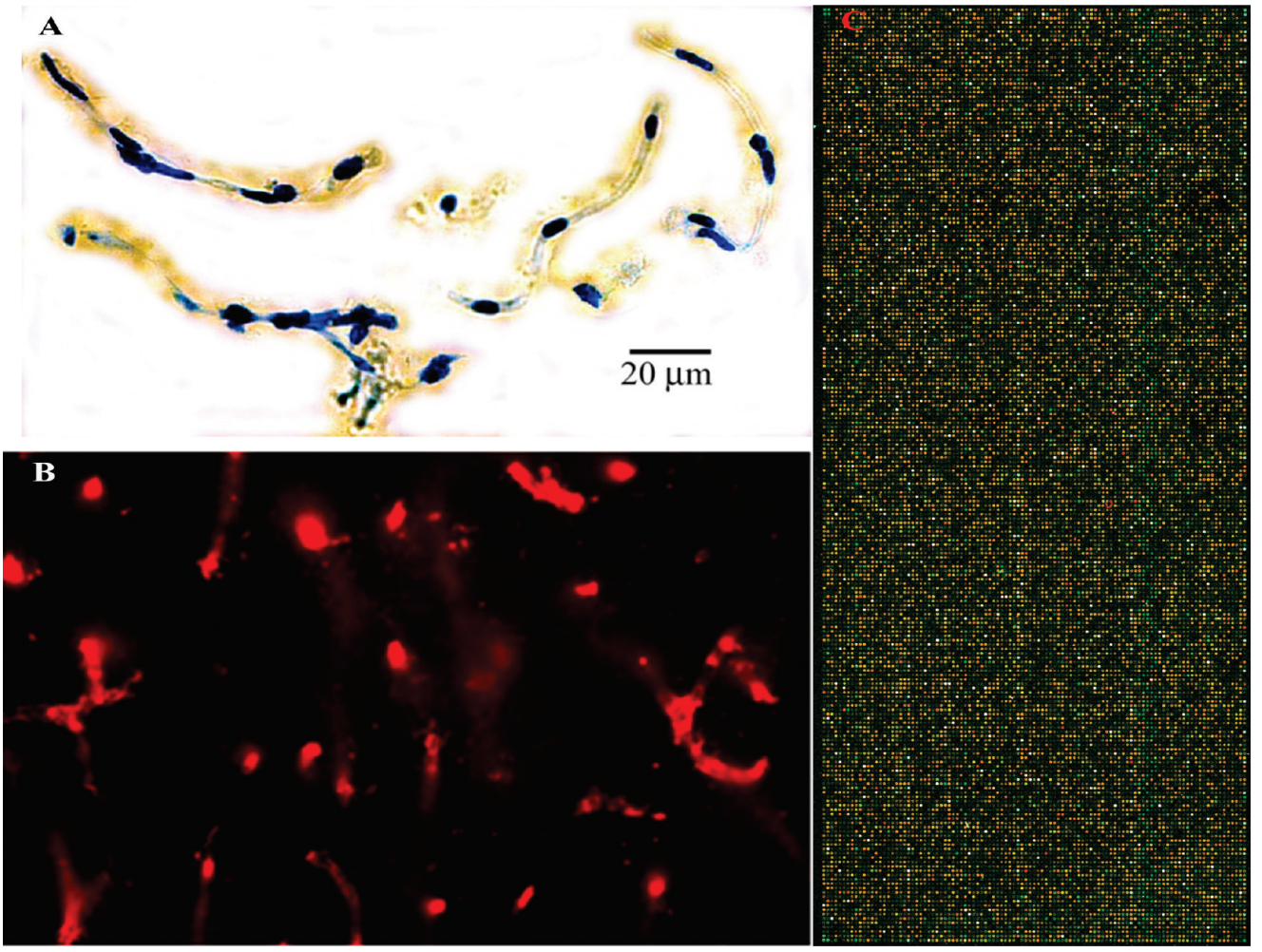

Figure 1. Isolation of brain microvessels and hybridization of microarrays. Microvessels were isolated using a filtration method and stained with toluidine blue (A). Microvessels in the brain are delineated in situ using RECA-1 immunoreactivity and are similar to those isolated by the filtration method (B vs A). Microarrays with 22 000 transcripts (C) were used to demonstrate that there are 16 PDE transcripts in the PDE superfamily including PDE4D and PDE5A in microvessels (see Table 1).

PDEs also play a pivotal role in regulation of cerebral blood flow (CBF) at both physiological and pathophysiological states. Noticeably, the cyclic nucleotide-related signal transduction pathways are particularly important to the vasodilating function of cerebral arteries and arterioles.[15] Mechanistically, a basic level of NO produced in the endothelium activates the soluble guanylate cyclase to generate cGMP. The elevated cGMP inhibits PDE3 and may in turn lead to an increase in intracellular cAMP.[16] In newborn pigs, PDE inhibitors applied topically dilated pial arterioles with concomitant increases in cAMP and/or cGMP levels in cerebral spinal fluid.[17] Furthermore, cAMP and/or cGMP appear to be involved in arterial vasodilation in response to hypercapnia.[17] Regulation of PDEs on CBF may interact with the nitric oxide (NO) pathway and histamine receptor(s).[18,19] NO- and prostanoid-induced vascular relaxations largely rely on cGMP and cAMP generation, respectively.[15] Regulation of the cell signaling pathways by PDEs may involve activation of protein kinases and ion channels as well as changes of intracellular Ca^{2+} in modulation of cerebral arterial tones.[17,20]

In the present study, we profiled gene expression in brain microvessels using microarray technology, highlighting PDE superfamily components. We used immunofluorescent markers to define the location of PDE4D and PDE5A in brain microvessels by costaining with an endothelial marker, rat endothelial cell antigen-1 (RECA-1), and a vascular machinery marker, α-smooth muscle actin (α-SMA). Apparently, defining the PDE superfamily components in brain microvessels would be an essential step facilitating future studies to explore their biological role and application value.

RESULTS AND DISCUSSION

Morphology of the Harvested Microvessels. Microvessels isolated from the cerebral cortex (Figure 1A) were relatively clean, and their morphology was comparable to microvessels observed in situ as delineated using RECA-1 immunoreactivity (Figure 1B). Microvessels isolated from the infratentorial area, however, included nonmicrovessel elements, presumptively ependymal cells (data not shown).

Determination of RNA Quality and Quantity and Representative Microarrays. The quality and quantity of RNA extracted from brain microvessels were verified using a Bioanalyzer instrument and quantitative real time PCR. Total RNA amounts were 2.72 ± 0.75 and 0.25 ± 0.04 μg, respectively, in microvessels

Table 1. Phosphodiesterase Gene List for Brain Microvessels in Rats ($n = 2$) (Microarray Outcomes among 22 000 Transcripts)[a]

gene ID[b]	*pde* transcripts	biochemical characteristics	density ratio IT vs CX	*P*-value
A_43_P12899	*pde6gamma*	cGMP-specific	**1917:2171**	0.60
A_43_P12503	*pde2a2*	cGMP simulated	20908:56993	<0.01
A_43_P15361	*pde9a*	cGMP-specific	2062:1507	0.39
A_43_P11785	*pde4d*	cAMP-specific	**1141:1376**	0.47
A_43_P19102	*pde4b*	cAMP-specific	1826:2419	0.53
A_43_P11870	*pde3b*	cGMP inhibited	2374:1938	0.49
A_43_P12310	*pde1b*	Ca_2^+calmodulin dependent	2492:3006	0.39
A_43_P13729	*pde1b1*	Ca_2^+calmodulin dependent	3546:3895	0.68
A_43_P11923	*pde3a*	cGMP inhibited	1097:973	0.73
A_43_P13092	*Pde7b*	cAMP-specific	1890:1335	0.38
A_43_P15462	*pde11a*	Hydrolyse both cAMP and cGMP	1034:1650	0.14
A_43_P15466	*pde5a*	cGMP-specific	**1503:1218**	0.54
A_43_P11712	*pde4a*	cAMP-specific	8824:9782	0.57
A_42_P584045	*pde10a3*	Testis-specific	3309:4944	0.09
A_43_P12467	*pde1a*	Ca_2^+calmodulin dependent	1848:2407	0.25
A_42_P628967	*Pde7-1*	cAMP-specific	2549:2831	0.50

[a] Microarrays with 22 000 transcripts demonstrate that there are 16 PDE transcripts in the PDE superfamily that are expressed in microvessels. Two cortical total RNA samples, each from different animals, were independently processed for amplification/fluorescent cRNA synthesis and hybridization microarrays against the infratentorial RNA samples following the protocol provided by the manufacturer. [b] Gene ID was provided by the microarray manufacturer (Agilent Technologies, Palo Alto, CA). IT, infratentorium; CX, cortex.

derived from the cerebral cortex and from the infratentorial brain blocks. The acquired microarray images (Figure 1C) demonstrated that the yellow spots (cyanine 5 labeled as red and cyanine 3 labeled as green) accounted for the most of the colored spots and they were evenly distributed over the whole image without significant spot deficit, indicating that the majority of the genes were unchanged.

Microarray Outcomes. Microarrays with 22 000 transcripts demonstrated that there were 16 PDE transcripts in the PDE superfamily showing quantifiable density (arbitrary density ranging between 973 and 56 993) in microvessels (see Table 1). The PDE isoforms showing the greatest expression in vascular smooth muscle are PDE1, PDE3, PDE4, and PDE5.[21] The gene list identified in the present study is consistent with the above report, demonstrating that levels of *pde1a*, *pde1b/pde1b1*, *pde3a*, *pde3b*, *pde4a*, *pde4b*, *pde4d*, and *pde5a* are quantifiable. In addition, the list from the present study includes seven other PDE components: *pde2a2*, *pde6gamma*, *pde7-1*, *pde7b*, *pde9a*, *pde10a3*, and *pde11a*. It has been recently reported that almost all PDE components more or less expressed in human brain,[22] and the present study extended these findings by demonstrating 16 PDE components in brain microvessels.

As categorized,[1] the PDEs that hydrolyze both cyclic nucleotides include PDE1C; PDE2A; PDE3A, B; PDE10A; PDE11A. The cAMP-specific PDEs are PDE4A, B, C, D; PDE7A, B; and PDE8A, B. The remaining PDEs (PDE5A; PDE6A, B, C; and PDE9) are classified as cGMP-specific. The gene list identified in the present study includes five that are cAMP-specific and three that are cGMP-specific and suggests that the functional regulation of brain microvessels by members of the PDE superfamily is complex. Since reliability is an important requirement for a control system, the built-in redundancy of PDEs in brain microvessels may serve to guarantee reliability in the regulation of vascular function. In similar fashion, control of rereplication of DNA depends on a redundant mechanism in which negative regulation of DNA replication factor Cdt1 functions in parallel with the negative regulation of prereplication complex component Cdc18 to ensure that the genome is duplicated exactly once each cell cycle.[23] On the other hand, the seemingly "redundant" expression of PDEs in brain microvessels may also be attributed to compartmentalization: it is widely accepted that cellular cyclic nucleotide signaling is compartmentalized and there is increasing evidence that PDEs are selectively distributed in subcellular compartments.[24−27] A single cell type can express several different PDEs and the nature and localization of these PDEs is likely to be of major regulatory importance for the local intracellular concentrations of cAMP and/or cGMP.[27] Localized PDEs may modulate the three-dimensional shape, amplitude, and temporal duration of cyclic nucleotides in selective cellular compartments.[27]

In total, 246 transcripts displayed differential expression ($p < 0.05$, 2-folds). Noticeably, a number of these transcripts were relevant to cell growth: *Rattus norvegicus* platelet-derived growth factor receptor beta (Pdgfrb); platelet-derived growth factor A-chain (PDGF A-chain); transforming growth factor beta stimulated clone 22; *Rattus norvegicus* VGF nerve growth factor; stromal cell-derived factor 1; transcription elongation factor A2; transcription elongation factor A (SII); and Kruppel-like factor. Among 16 members of the PDE superfamily that displayed substantial density, PDE2a2 was differentially expressed. Interestingly, there is ∼10-fold difference between the cerebral cortex and cerebellum in *pde2* mRNA level from pooled human brain tissues.[22] The present microarray outcome is consistent with this report, showing a >2-fold density difference in *pde2a2* expression between the cerebral cortical microvessels and the microvessels collected from infratentorium in rats.

It is worth mentioning that the filtration method employed in the present study may only be appropriate for isolating comparatively pure microvessels from the cerebral cortex because the isolated microvessels from some brain regions, such as the infratentorial region, may contain other cellular components such as ependymal cells. The higher transcript expression in the infratentorial brain block as demonstrated by the microarray data

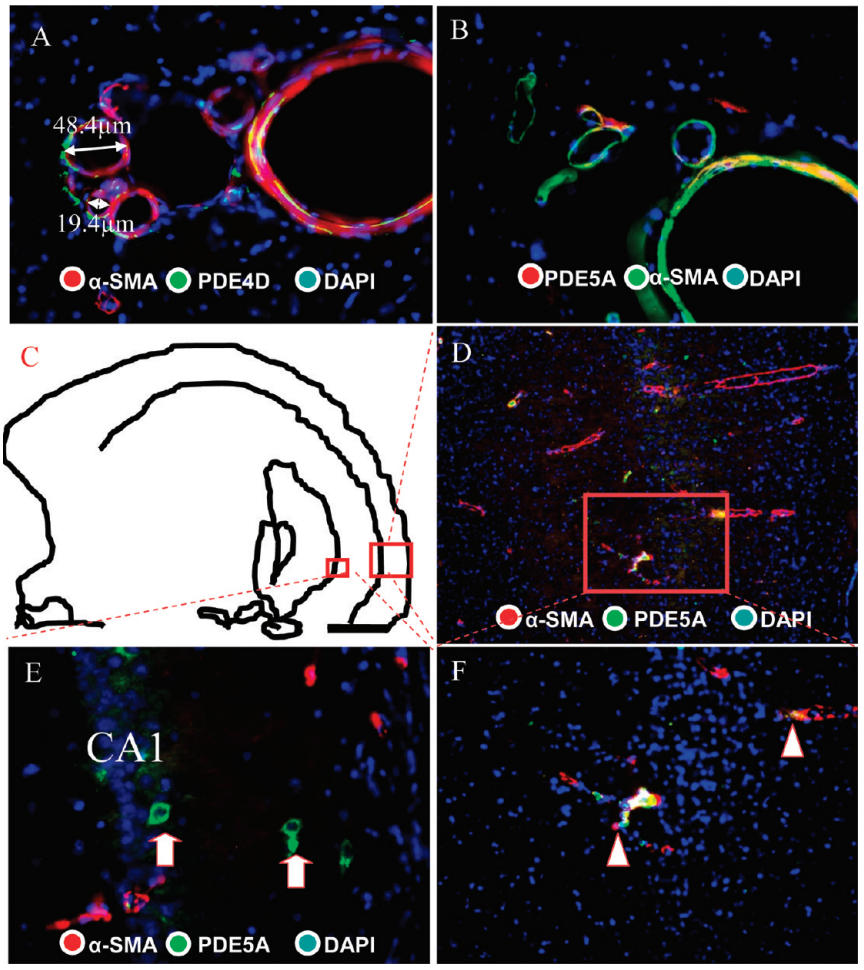

Figure 2. Protein expression of PDE4D and PDE5A in brain microvessels: coimmuno-staining with α-SMA. PDE4D (A) and PDE5A (B) immunoreactivities are colocalized in the cortical blood vessels with α-SMA immunostain that delineates vasculature morphology. Some cortical pericytes express PDE5A (D and F). The pericytes (arrows) are identified by their morphological features as delineated by α-SMA immunoreactivity: ~10 μm is the diameter of the vessel-like structure and single layer of smooth muscle cells. Interestingly, a few cells (arrows) in the hippocampal CA1 region express PDE5A proteins (E). Illustration in (C) demonstrates the anatomic outline of a semihemisphere section across the middle hippocampus. The red squares in (C) indicate the locations of image acquirement, hippocampal CA1 region (E) and cortical regions (D and F), while images (A) and (B) were acquired along the cerebral longitudinal fissure. Hipp, hippocampus; CX, cortex.

argues that the ependymal cells were included in this analysis because they exhibit features of neural stem or progenitor cells.[28,29]

Immunofluorescent Studies. Immunoreactivities of either PDE4D or PDE5A were comparatively appreciable in vasculatures of rather large size. As demonstrated in Figure 2A and B, PDE4D or PDE5A, colocalized with α-SMA, was readily found in the vessels located along the longitudinal cerebral fissure. Among those vessels, some were less than 100 μm in diameter and were suited for definition as microvessels. Co-immunoreactivity of PDE5A with α-SMA led to the conclusion that pericytes, delineated by the α-SMA labeling and defined by their single layer of muscular cells and small size (~10 μm in diameter), expressed PDE5A (Figure 2D and F). There was little PDE5A signal in the bran parenchyma other than in vasculature, while a few cells in the hippocampal CA1 region expressed PDE5A (Figure 2E). The present study failed to demonstrate that pericytes expressed PDE4D. However, many brain cells such as the hippocampal CA1 neurons expressed PDE4D (data not shown).

RECA-1 delineated capillaries very well (Figures 3 and 4). PDE5A (Figure 3A−D) and PDE4D (Figure 3E−H) immunostains were found to be colocalized with RECA-1 immunoreactivity. Three-dimensional images reconstructed from stacked images showed cut views and surface-rendered fluorescence to verify the colocalization of PDE4D with RECA-1 (Figure 4).

PDE4 merits particular attention because it has been reported to represent the principal cAMP hydrolyzing enzyme in cerebral vascular smooth muscle.[30] Noticeably, PDE4D has received a lot of attention clinically because of its ability to influence vulnerability to stroke.[31−33] On the other hand, PDE5 is of interest because it is cGMP-specific and also because the selective PDE5 inhibitor, sildenafil (Viagra), is widely used in clinics to treat erectile dysfunction and pulmonary arterial hypertension. One of the side effects of sildenafil is headache that occurs even in healthy subjects;[34,35] however, the pathogenic mechanism(s) underlying sildenafil-induced headache remains unknown. Because cerebral hemodynamic responses and neuron excitability are not affected by sildenafil[34,35] and also because the known physiological functions of PDEs include regulation of vascular tone and permeability, the sildenafil-induced headache is plausibly ascribable to alterations in the integrity of the BBB since sildenafil (PDE inhibitor) may

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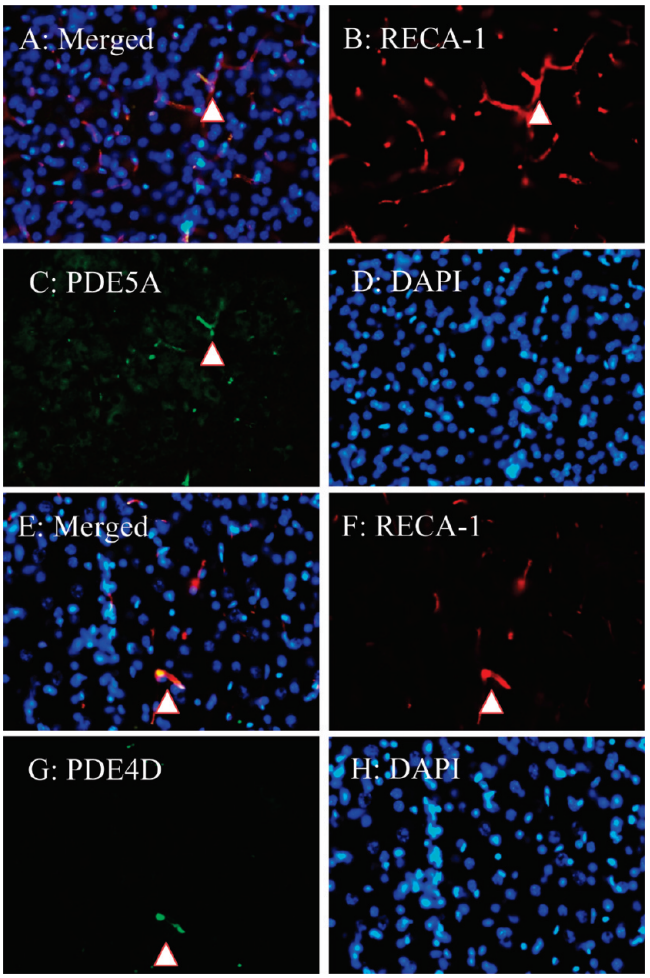

Figure 3. Protein expression of PDE4D and PDE5A in brain microvessels: coimmunostaining with RECA-1. PDE5A (A−D) and PDE4D (E−H) immunoreactivities are seen in cortical blood vessels (identified by coimmunostaining with RECA-1 that delineates vessel morphology).

alter vascular PDE expression.[36] The knowledge gained from the present study may have applicability for both the clinic and research laboratory, since it demonstrates that PDE4D immunoreactivity is found in capillaries (costained with the endothelial marker, RECA-1) as well as in comparatively large brain vessels. Possibly, the integrity of the BBB as influenced by PDE4D control may play a role in stroke susceptibility. Moreover, the present study demonstrates that PDE5A proteins are expressed in the cerebral microvessel pericytes, a key component in the control of microcirculation and the BBB,[37−39] as defined by the α-SMA labeling[40] and size and contour of the microvessels.

In conclusion, the filtration method provides appropriate microvessel preparations from the cerebral cortex that provide suitable RNA for genome-wide microarray analyses, highlighting PDE superfamily components. These methods may serve to help identify sensitive biomarker(s) of brain microvessel function and allow for the detection of subtle molecular changes in the brain microvessels in response to physiological and pathophysiological stimuli.

METHODS

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee. Eight adult male Fischer 344 rats were decapitated, and brains were harvested immediately on an ice-cooled metal platform. Cerebral cortex and infratentorial brain blocks from seven rats were dissected for microvessel isolation. One brain sample was fixed in buffered formalin, cut into 30 μm thick coronal sections with a sliding microtome, and processed for immunofluorescent staining of RECA-1 to compare morphology between the filter isolated microvessels and microvessels in situ.

Spared slices, 30 μm thick, free-floating in 1× phosphate buffer solution (PBS) containing 0.02% sodium azide, generated in other projects were used for verification of the targeted PDE proteins explored in microarrays. These slices included cerebral and cerebellar samples from adult Fischer 344 rats (n = 3 each) and from weaning and adult Sprague−Dawley rats (n = 3−6 each age).

Microvessels. A filtration method was used to isolate brain microvessels (at 4 °C) as described with slight modification.[41,42] Briefly, the cerebral cortex and infratentorial brain blocks were minced on an ice-cold aluminum plate, homogenized, and transferred to the interior of a 210 μm Nytex bag submerged in ice-cold isotonic PBS buffer containing glucose and sucrose. The tissue then passed through the bag and was recollected by low speed centrifugation. The pellet was resuspended and filtered through an 80 μm nitrocellulose filter under a vacuum.

RNA Isolation and Quantity and Quality Determination. The vessels collected on the filter were washed with ice-cold buffer and then lysed for RNA isolation. A kit from Qiagen was used to purify the RNA. The quality and quantity for total RNA isolated from all samples were determined using a model 2001 Bioanalyzer (Agilent Technologies, Palo Alto, CA) instrument. Also, isolated RNA was evaluated by quantitative real time PCR (Q-PCR) for glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA to verify quality. An ABI Prism Sequence Detection System 7900 HT (Applied Biosystems, Foster City, CA) instrument and a commercially available GAPDH primer/probe set were used as described before.[43]

Microarray Analyses. Two-color, Cy3- and Cy5-labeled aRNA was hybridized to rat Agilent microarrays (~22 000 features, catalog number G410A) following the manufacturer's protocol, except that 1.0 μg of probe was used instead of 0.75 μg. Hybridization was conducted at 60 °C for 17 h with equal amounts of two labeled aRNAs. Two microarrays using four sample RNAs (microvessels from the cerebral cortex vs microvessels from the infratentorial block) were included in the present study. Two cortical total RNA samples, each from different animals, were independently processed for amplification/fluorescent cRNA synthesis and hybridization microarrays against the infratentorial RNA samples following the protocol provided by the manufacturer. Using Scanarray software, the image data were then converted into numerical data and stored in a text file. Each gene was marked as "present, absent or marginal" using its signal intensity data. Thereafter, the text files were imported into GeneSpring software (version 6.1, Silicon Genetics, Redwood City, CA) for microarray analysis. Lowess intensity dependent normalization was used for two-color data analysis. In addition, the GAPDH density values were referred in the normalization. Statistical treatment of ANOVA analysis followed by the nonparametric Wilcoxon−Mann−Whitney test was performed to establish a gene list in which each gene had a p-value less than 0.05 (2-fold change). To highlight the genes of interest, we focused on the PDE superfamily components to establish a list of genes that displayed quantifiable spots on microarray images.

Histological Assessments. *Methyl Green Staining/Toluidine Blue Staining.* The materials collected using the filtering method as described before were stained with methyl green/toluidine blue solution. Briefly, the materials together with the collecting 80 μm nitrocellulose filter, derived from either the cerebral cortex or the infratentorium, were fixed in 1 mL of 10% buffered formalin for 24 h. After vortexing for 30 s, the formalin-material solution was dropped to a glass slide and then dried for 1 h in 60 °C. The microvessels were stained using 1% methyl

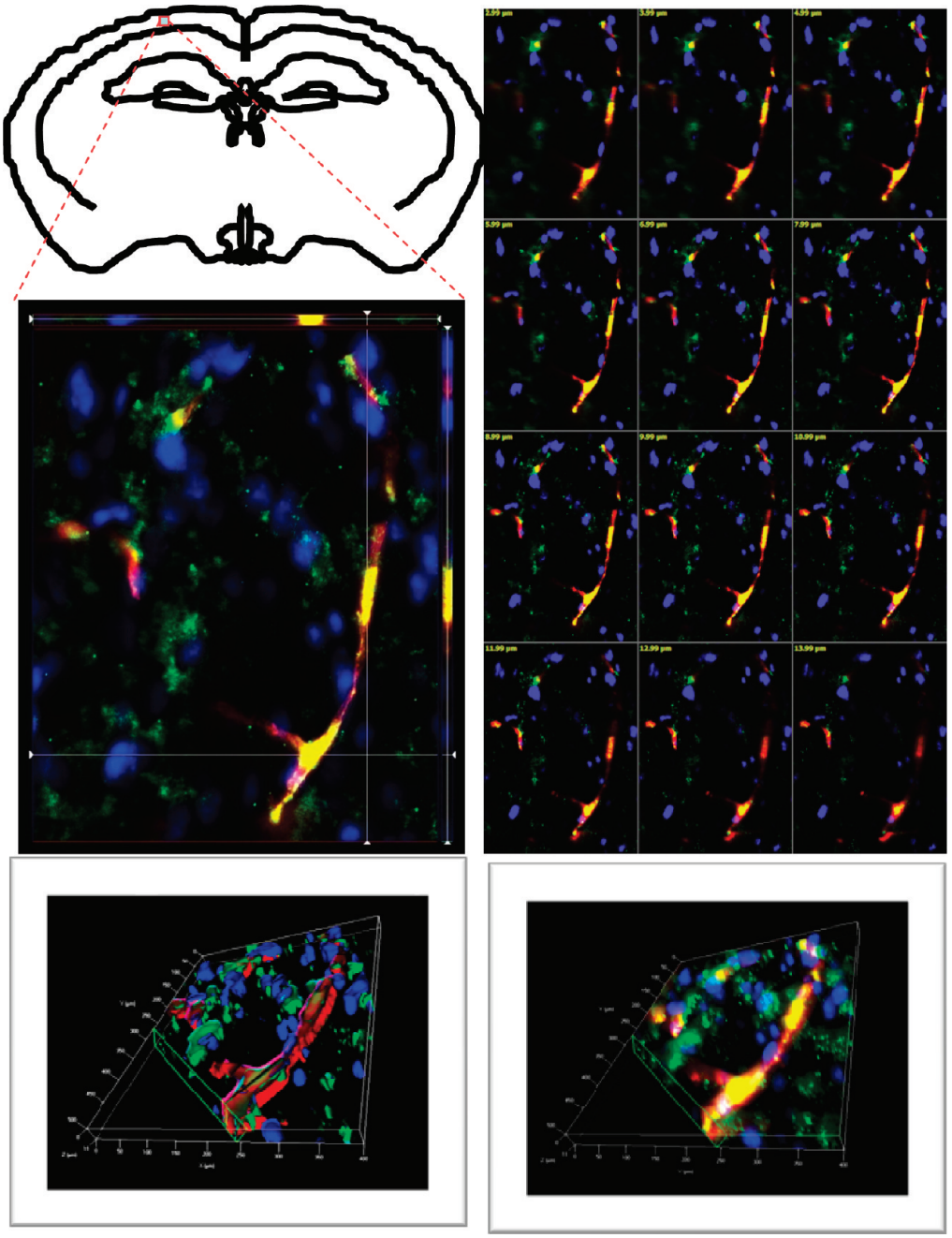

Figure 4. Protein expression of PDE4D in brain microvessels: coimmunostained with RECA-1 in three-dimensional images. The panels show the cut view of a single slice (middle left panel) and multiple slices (upper right panel), the surface-rendered fluorescence from stacked slices for PDF4D antigen (green) and RECA-1 (red) (lower left panel), and for PDE4D (yellow fluorescence) and RECA-1 (lower right panel).

green or 2% toluidine blue and washed before images were acquired under a microscope.

Immunofluorescent Studies. *Antibodies.* Rabbit polyclonal antibodies for PDE4D and PDE5A were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies for RECA-1 and α-SMA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Neomarkers (MS-113-P1, Fremont, CA), respectively. Secondary antibodies conjugated with fluorescent Alexa 488 and Alexa 594 were purchased from Molecular Probes (Eugene, OR).

Triple Labeling of PDE4D/PDE5A, RECA-1/α-SMA, and DAPI. A modified triple labeling method was developed as previously described.[44−49] Briefly, free-floating, 30 μm thick slices were washed three times with PBS before being hydrated with Milli-Q water. For antigen retrieval, the slices were immersed in citrate buffer (10 mM, pH 6.0) and heated in a microwave to 100 °C. Thereafter, the slices were cooled down slowly with Milli-Q water and then washed again with PBS before application of normal goat serum-PBS (4%) to inhibit nonspecific binding. Primary antibodies were diluted in the following ratios: PDE4D/PDE5A, 1:200; RECA-1, 1:300; α-SMA, 1:400. These were incubated with sections for 72 h at 4 °C. The secondary antibodies were diluted to 1:200 either for Alexa 488-labeled/Alexa 594-labeled goat anti-mouse antibodies or for Alexa 594-labeled/Alexa 488-labeled goat anti-rabbit antibodies; they were incubated with sections for 75 min at room temperature. 4′,6-Diamidino-2-phenylindole (DAPI) was utilized for visualizing nuclei.

Images for PDE4D and PDE5A or RECA-1 and α-SMA immunoreactivity were acquired in different camera channels using filters for fluorescein isothiocyanate or Texas red and DAPI, respectively. Using AxioVision version 4.7 software (Carl Zeiss MicroImaging, Inc. NY), three-dimensional images were reconstructed using stacked images and showing cut views and surface-rendered fluorescence.[50]

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Author Contributions

Z.H. designed and conducted all experiments, and wrote the manuscript; L.C. performed microarray data analysis and 3-dimensional image reconstruction; T.A.P. reconfirmed RNA quality and microarray data analysis and revised the manuscript; M.G.P. contributed to the conceptual design and significantly edited the manuscript.

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DISCLOSURE

This document has been reviewed in accordance with United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect the position or opinions of the FDA nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the FDA.

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